

NAG-8-1354  
Univ. AL Huntsville

**FY 01 PI Data Update Form**  
**Physical Sciences Division**

*Final Report*

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**Task Research Title**

Protein-Precipitant-Specific Criteria for the Impact of Reduced Gravity on Crystal Perfection

<i>Monitoring Center</i>	MSFC	<i>NAG Number</i>	NAG8-1354
<i>Research Type</i>	Ground	<i>Discipline</i>	Biotechnology
<i>Initiation Date</i>	Jun-97	<i>Expiration Date</i>	11/01

Degree Kind	Students Funded	Degrees Granted
BS	1	0
MS	1	0
PhD	1	0
<b>Totals</b>	<b>3</b>	<b>0</b>

Co-Investigator Name	Co-Investigator Affiliation
1. Thomas, B.R.	Universities Space Research Association
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**Acronyms** (Please list and define any acronyms associated with your project)

## **Impact on America**

Insight into the crystallization processes of biological macromolecules into crystals or aggregates can provide valuable guidelines in many fundamental and applied fields. Such insight will prompt new means to regulate protein phase transitions *in-vivo*, e.g., polymerization of hemoglobin S in the red cells, crystallization of crystallins in the eye lens, etc. Understanding of protein crystal nucleation will help achieve narrow crystallite size distributions, needed for sustained release of pharmaceutical protein preparations such as insulin or interferon. Traditionally, protein crystallization studies have been related to the pursuit of crystal perfection needed to improve the structure details provided by x-ray, electron or neutron diffraction methods. Crystallization trials for the purposes of structural biology carried out in space have posed an intriguing question related to the inconsistency of the effects of the microgravity growth on the quality of the crystals.

## **Industrial Affiliates**

**Please list any industry research contacts you may have**

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Dr. Daniel Carter, New Century Pharmaceuticals, Inc.

**If this investigation has contributed to the development of any new technological advances, please identify each one and include a short description.**

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Apparatus for protein crystal growth under forced solution flow  
Temperature-controlled cell for interferometric in-situ monitoring of protein crystals growing from flowing solutions

Differential phase-shifting interferometry for studies of step pattern evolution during solution growth

**Who is using the results of your research?**

New Century Pharmaceuticals, Inc.  
Protein crystal growers, both in industry and academia

**Where have your recent graduate students found employment?**

University of Alabama in Huntsville

**Number of times that your work has appeared in the popular press?** 3

**Number of times that your work has appeared on a magazine cover?** 2

**If you have a science website, or your work is represented on one, please include the address:**

[www.chee.uh.edu/vekilov](http://www.chee.uh.edu/vekilov)

<http://unisci.com/stories/20003/0803004.htm>

<http://www.lahaaland.com/science/physics/physics255.html>

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## **Task Objective**

The objective of this research is to provide quantitative criteria for the impact of reduced or enhanced convective transport on protein crystal perfection. Our earlier work strongly suggests that the magnitude of (lattice defect-inducing) fluctuations in the crystallization rate of proteins arise from the coupling of bulk transport and nonlinear interface kinetics. Hence, we surmised that, depending on the relative weight of bulk transport and interface kinetics in the control of the crystallization process on Earth, these fluctuations can either increase or decrease under reduced gravity conditions. The sign and magnitude of these changes depend on the specific protein-precipitant system. As a consequence, space environments can be either beneficial or detrimental for achieving structural perfection in protein crystals. The task objectives consist in systematic investigations of this hypothesis.

## **Task Description**

Criteria for optimizing transport conditions with respect to resulting crystal perfection will be derived from numerical simulations of coupled bulk-transport and step-kinetics in protein solution growth. Specific kinetics and transport input parameters will be experimentally determined for select proteins for which the diffraction results indicate either higher or lower perfection of the space-grown crystals. These enabling activities will include compositional characterization and purification of the proteins; determinations of the temperature dependence of solubilities for certain pH and precipitant conditions; and, determinations of step kinetics coefficients and step bunching under natural and forced flow conditions. Assuming that the simulation results for a specific protein-precipitant system reproduce the experimentally observed ground-based step dynamics behavior, high-fidelity predictions of corresponding low-gravity and forced-flow behaviors will be made. The relevance of these considerations for lattice perfection will be tested by evaluations of the x-ray diffraction resolution resulting from ground-based crystallization under select combinations of transport and kinetics conditions.

## **Task Significance**

The pharmaceutical industry needs protein structure information to facilitate rational drug design. However, many of the currently available protein crystals are too imperfect to yield sufficiently detailed structure information. The significance of this research lies in the expected system-specific, quantifiable criteria for the choice of either reduced gravity or enhanced bulk transport conditions for achieving improved (protein) crystal quality. Such criteria do not exist at this point.

## **Task Progress**

**Growth Dynamics of Hemoglobin C Crystals.** The mutated hemoglobin, HbC ( $\beta 6$  Glu $\rightarrow$ Lys), in the oxygenated (R-) liganded state, forms crystals inside red blood cells of patients with CC and SC diseases. Using a novel miniaturized light-scintillation technique, we quantified a strong retrograde solubility dependence on temperature. The data were used to select a longitudinal temperature gradient in a crystallization capillary that yielded an X-ray diffraction limit of 1.8 Å, an improvement of 0.2–0.3 Å. Static and

dynamic light scattering characterization of the intermolecular interactions between the R-state (CO) HbC, HbA and HbS molecules in solution showed that (1) electrostatics is unimportant and (2) the interactions are dominated by the specific binding of solutions' ions to the proteins. Microscopic observations and determinations of the nucleation statistics showed that the crystals of HbC nucleate and grow by the attachment of native molecules from the solution and that concurrent amorphous phases, spherulites and microfibrils, are not building blocks for the crystal. Thermodynamic analyses of HbC crystallization based on the retrograde solubility yielded a high positive enthalpy of  $155 \text{ kJ mol}^{-1}$ , i.e., the specific interactions favor HbC molecules in the solute state. Then, HbC crystallization is only possible because of the huge entropy gain of  $610 \text{ J mol}^{-1}\text{K}^{-1}$ , likely stemming from the release of up to 10 water molecules per protein intermolecular contact—hydrophobic interaction. Thus, the higher crystallization propensity of R-state HbC is attributable to increased hydrophobicity resulting from the conformational changes that accompany the HbC  $\beta 6$  mutation.

We investigated the molecular-level mechanisms of crystallization of this protein in its stable carbomonoxy form using high-resolution atomic force microscopy. We found that in conditions of equilibrium with the solution, the crystals' surface reconstructs into four-molecule wide strands along the crystallographic **b** axis. However, the crystals do not grow by the alignment of such preformed strands. We found that the crystals grow by the attachment of single molecules to suitable sites on the surface. These sites are located along the edges of new layers generated by two-dimensional nucleation or by screw dislocations. The density of the growth sites is determined by the free energy of crystallization that we have independently determined. During growth, the steps propagate with random velocities. At higher supersaturations, the density of steps varies in a broad range and this could result in crystal non-uniformity. At high concentrations of HbC, aggregates form in the solution, adsorb on the surface, and hinder growth, indicating that crystal quality improvements should be sought at conditions that inhibit aggregation and do not induce excessive step density non-uniformity.

**Are protein crystallization mechanisms relevant to understanding and control of polymerization of deoxyhemoglobin S?** We investigated the homogeneous nucleation and subsequent evolution of polymers of sickle cell hemoglobin (HbS) using differential interference contrast (DIC) microscopy. The same technique was employed to determine the liquid-liquid separation boundaries for a variety of conditions in solution of sickle cell and normal human hemoglobin. The HbS polymers were also imaged using atomic force microscopy. We found that the location of this phase boundary under conditions that mimic those in the erythrocytes is consistent with previous determinations of the spinodal for this phase transition. Increasing the ionic strength shifts this phase boundary to significantly lower temperatures and Hb concentrations. We also found that the nucleation of individual HbS fibers indicates that the process is random and follows statistics similar to those established for nucleation of crystals or liquid droplets from vapors.

**Thermodynamics and interactions in aqueous solutions of proteins.** We used chromatographic, static and dynamic light scattering techniques, and atomic force microscopy (AFM) to study the structure of the protein species and the protein-protein interactions in solutions containing two apoferritin molecular forms, monomers and dimers, in the presence of NaAc buffer and  $\text{CdSO}_4$ . The sizes and shapes of the

monomers and dimers, separated by size-exclusion chromatography, were determined by dynamic light scattering and AFM. While the monomer is an apparent sphere with a diameter corresponding to previous x-ray crystallography determinations, the dimer shape corresponds to two, bound monomer spheres. Static light scattering was used to characterize the interactions between solute molecules of monomers and dimers in terms of the second osmotic virial coefficients. The addition of even small amounts of  $\text{Cd}^{2+}$  causes strong attraction between the monomer molecules, but does not lead to oligomer formation, at least at the protein concentrations used. Furthermore, we found that the second virial coefficient and the protein solubility do not noticeably depend on temperature in the range from 0 to 40 °C. This suggests that the enthalpy for crystallization of apoferritin is close to zero, and the gain of entropy is essentially constant in this temperature range. We also found that in solutions of the apoferritin dimer, the molecules attract even in the presence of acetate buffer only, indicating a change in the surface of the apoferritin molecule. In view of the repulsion between the monomers at the same conditions, this indicates that the dimers and higher oligomers form only after partial unfolding of some of the apoferritin subunits. These observations suggest that aggregation and self-assembly of protein molecules or molecular subunits may be driven by forces other than those responsible for crystallization in the protein solution.

**Bibliography: Please list citations for FY 2001 only (sample below)**

**FY01 BIBLIOGRAPHIC CITATIONS  
NASA PHYSICAL SCIENCES**

**Book Chapters**

1. P.G. Vekilov, *Cover Pictures and Story*, in **Advances in Crystal Growth Research**, edited by K. Sato, Y. Furukawa, and K. Nakajima. (Elsevier, Amsterdam, 2001).

**Journals**

1. R. E. Hirsch, R.E. Samuel, N.A Fataliev, M.J. Pollack, O. Galkin, P.G. Vekilov, and R.L. Nagel, *Differential pathways in oxy and deoxy HbC aggregation/crystallization*. *Proteins* **42** (2000), 99-107.
2. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for the self-assembly of apoferritin molecules into crystals*. *J. Mol. Biol.* **303** (2000) 667-678.
3. S.-T. Yau and P.G. Vekilov, *Direct observation of nucleus structure and nucleation pathways*, *J. Am. Chem. Soc.* **123** (2001) 1080-1089.
4. S.-T. Yau, B.R. Thomas, O. Galkin, O. Gliko, and P.G. Vekilov, *Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization*, *Proteins* **43** (2001) 343-352.
5. M.D. Serrano, O. Galkin, S.-T. Yau, B.R. Thomas, R.L. Nagel, R. E. Hirsch, and P.G. Vekilov, *Are protein crystallization mechanisms relevant to understanding and control of polymerization of deoxyhemoglobin S?* *J. Crystal Growth* **232** (2001) 368-375.
6. D.N. Petsev, B.R. Thomas, S.-T. Yau, D. Tsekova, C.N. Naney, W.W. Wilson, and P.G. Vekilov, *Temperature-independent solubility and interactions between apoferritin monomers and dimers in solution*. *J. Crystal Growth* **232** (2001) 27-29.

### **Invited presentations**

1. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Phase transitions in protein solutions: structures, dynamics and control pathways*. 2000 Biology Retreat, Guntersville, Alabama, USA, September 29-30, 2000.
2. P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport*. Texas Christian University, Dallas, Texas, November 2, 2000.
3. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Structures, dynamics and control pathways of protein crystal nucleation*. Southern Methodist University, Fort Worth, Texas, November 3, 2000.
4. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *Real-time in situ monitoring with molecular resolution of the elementary processes of crystallization of apoferritin*. 2000 Annual Meeting of the American Institute of Chemical Engineers, Los Angeles, California, November 12-17, 2000.
5. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *What do we learn about biological molecules from watching them partake in phase transitions?* Seminar, Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, Alabama, USA, November 29, 2000.
6. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into crystals?* Seminar, Hokaido National Industrial Research Institute, Sapporo, Japan, December 8, 2000.
7. P.G. Vekilov, S.-T. Yau, H. Lin, D. Petsev, B. Thomas *Characteristic lengthscales of the protein crystallization processes: where can gravity affect growth*, Japan Space Utilization Promotion Center Tokyo, Japan, December 12, 2000.
8. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into protein crystals?* Seminar, Tohoku University, Sendai, Japan, December 13, 2000.
9. P.G. Vekilov, O. Galkin, S.-T. Yau, M. Wu, D.N. Petsev, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies*. Department of Chemical Engineering, University of Illinois, Champaign, IL, February 1, 2001
10. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Tracking Individual Molecules as They Attach Themselves to Crystals: Statistics, Dynamics and Mechanisms*. Physics Colloquium, University of Alabama in Huntsville, Huntsville, Alabama, February 7, 2001.
11. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N. Petsev, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies*. Department of Chemical Engineering, University of Houston, February 16, 2001
12. P.G. Vekilov, *Molecular mechanisms of crystallization of proteins*. Marshall Space Flight Center, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, February 28, 2001.
13. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N. Petsev, B.R. Thomas, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies*, University of Alabama in Huntsville, Research Council Meeting, Huntsville, Alabama, April 2, 2001. P. G. Vekilov, D.N. Petsev, S.-T. Yau, and K. Chen, *Crystallization of Small and Large Molecules*, 9th Inhalation Technology Seminar, Orion Pharma, Espoo, Finland, June 6, 2001

15. P.G. Vekilov, *Mechanisms of crystallization from solution: a short course*. VTT (Technology Research Center of Finland) Helsinki, Finland, June 7-8, 2001.
16. P.G. Vekilov, O. Galkin, M. Wu, K. Chen, *Phase transition in protein solutions: dynamics and control strategies*; Colloquium, Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, June 12, 2001.
17. P.G. Vekilov, O. Galkin, D.N. Petsev, M. Wu, *Dynamics of phase transition in proteins solutions*, Albert Einstein College of Medicine, Department of Medicine, Division of Hematology, The Bronx, NY, June 27, 2001.
18. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level parameters for the self assembly of biological macromolecules into crystals*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
19. O. Galkin and P.G. Vekilov, *Liquid-liquid separation in solutions of proteins: implications for the formation of condensed phases*. 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
20. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Molecular-resolution atomic force microscopy movies of step propagation around surface defects and impurities*. 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
21. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization*. 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
22. P.G. Vekilov, S.-T. Yau, and H. Lin, *Characteristic lengthscales of the protein crystallization processes: where can gravity affect growth*. 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.

#### **Contributed Presentations**

1. S.-T. Yau and P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport*, First International Symposium on Microgravity Research and Applications, Sorrento, Italy, September 10-15, 2000.
2. A.R. Feeling-Taylor, S.-T. Yau, D.N. Petsev, O. Galkin, R. Nagel, R.E. Hirsch, and P.G. Vekilov, *Molecular Mechanisms of Crystallization of HbC*, 25th Annual Meeting National Sickle Cell Disease Program, New York, NY, April 13 - 17, 2001.
3. O. Galkin, K. Chen, R. Elison Hirsch, R.L. Nagel and P.G. Vekilov, *Liquid-liquid Separation in Solutions of Hemoglobins S and A: Implications for the Polymerization of HbS*, 25th Annual Meeting National Sickle Cell Disease Program, New York, NY, April 13 - 17, 2001.
4. H. Lin, S.-T. Yau, O. Gliko, and P.G. Vekilov, *Dynamics of Trains of Non-interacting Steps Growing under Diffusion Control*, 2001 Spring Materials Research Society Meeting, San Francisco, CA, April 16-21, 2001
5. S.-T. Yau, P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
6. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Phase transition in protein solutions: dynamics and control strategies*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001

7. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for crystallization*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
8. O. Galkin, D.N. Petsev, P.G. Vekilov, *Phase transition in protein solutions: dynamics and control strategies*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
9. S.-T. Yau, P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
10. P.G. Vekilov, S.-T. Yau, B.R. Thomas, O. Galkin, O. Gliko, and H. Lin, *Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization* 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
11. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level Thermodynamic and Kinetic Parameters for Crystallization*. 13<sup>th</sup> International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
12. P.G. Vekilov and O. Galkin, *Phase transition in protein solutions: dynamics and control strategies*, Keck Center 2001 Annual Research Conference, Galveston, TX, USA, September 21, 2001.